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The Reactivity of Thiol Groups and the Subunit Structure of Aldolase

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1. Seven unique carboxymethylcysteine-containing peptides have been isolated from tryptic digests of rabbit muscle aldolase carboxymethylated with iodo[2-^{14}C]acetic acid in 8M-urea. These peptides have been characterized by amino acid and end-group analysis and their location within the cyanogen bromide cleavage fragments of the enzyme has been determined. 2. Reaction of native aldolase with 5,5′-dithiobis-(2-nitrobenzoic acid), iodoacetamide and *N*-ethylmaleimide showed that a total of three cysteine residues per subunit of mol.wt. 40000 were reactive towards these reagents, and that the modification of these residues was accompanied by loss in enzymic activity. Chemical analysis of the modified enzymes demonstrated that the same three thiol groups are involved in the reaction with all these reagents but that the observed reactivity of a given thiol group varies with the reagent used. 3. One reactive thiol group per subunit could be protected when the modification of the enzyme was carried out in the presence of substrate, fructose 1,6-diphosphate, under which conditions enzymic activity was retained. This thiol group has been identified chemically and is possibly at or near the active site. Limiting the exposure of the native enzyme to iodoacetamide also served to restrict alkylation to two thiol groups and left the enzymic activity unimpaired. The thiol group left unmodified is the same as that protected by substrate during more rigorous alkylation, although it is now more reactive towards 5,5′-dithiobis-(2-nitrobenzoic acid) than in the native enzyme. 4. Conversely, prolonged incubation of the enzyme with fructose 1,6-diphosphate, which was subsequently removed by dialysis, caused an irreversible fall in enzymic activity and in thiol group reactivity measured with 5,5′-dithiobis-(2-nitrobenzoic acid). 5. It is concluded that the aldolase tetramer contains at least 28 cysteine residues. Each subunit appears to be identical with respect to number, location and reactivity of thiol groups.

Studies on the number of cysteine residues in rabbit muscle aldolase (EC 4.1.2.13) suggest that approximately 28 thiol groups are found per molecule of mol.wt. 160000 (Morse & Horecker, 1968, and references therein). Although it has been reported that the cysteine residues of aldolase are unequally distributed between two types of polypeptide chain (Chan, Morse & Horecker, 1967), recent evidence indicates that the subunits of aldolase have equal numbers of cysteine residues (Anderson, Gibbons & Perham, 1969). If a subunit mol.wt. of 40000 (Kawahara & Tanford, 1966) is assumed, it is probable that there are approximately 7 cysteine residues per subunit. One cysteine residue is known to occur very near in the primary structure to the substrate-binding lysine residue of aldolase (Lai, 1968). The location of the other cysteine residues within the primary structure is unknown.

The role of cysteine residues in rabbit muscle aldolase activity is also uncertain. Studies with *p*-mercuribenzoate (Swenson & Boyer, 1957; Szabolcsi & Biszku, 1961; Agatova & Emanuel, 1964) suggest that the thiol groups act to maintain protein conformation rather than to participate in the enzyme catalysis. However, studies with chlorodinitrobenzene, NEM* and $\beta\beta'$-carboxyethyl disulphide (Rowley, Tchola & Horecker, 1964; Kowal, Cremona & Horecker, 1965) and with DTNB (Eagles, Johnson, Joynson, McMurray & Gutfreund, 1969) suggest that a thiol group might occur at or near the active site, since substrate can prevent inhibition in all these cases, although the possibility that protection is afforded because of a conformational change when substrate is bound cannot be entirely excluded.

* Abbreviations: NEM, *N*-ethylmaleimide; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); FDP, fructose 1,6-diphosphate.

The present work was undertaken in the hope that further information on the number, location and function of thiol groups in aldolase might be obtained. It was also hoped that it might be possible to distinguish them by examining their reactions with various reagents.

MATERIALS AND METHODS

Reagents and enzymes. FDP (trisodium salt) was obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. DTNB, iodoacetic acid and iodoacetamide were obtained from British Drug Houses Ltd., Poole, Dorset, U.K. NEM was purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Cyanogen bromide, 2-mercaptoethanol and *N*-ethylmorpholine were obtained from Eastman Kodak, Kirkby, Lancs., U.K. The *N*-ethylmorpholine was redistilled before use. Iodo[2-^{14}C]acetic acid was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. and diluted with carrier iodoacetic acid to a specific radioactivity of 0.75mCi/mmol before use. Trypsin (twice recrystallized) was a product of Worthington Biochemical Corp., Freehold, N.J., U.S.A. All other chemicals were of analytical grade.

A modification (Gibbons & Perham, 1970) of the method of Taylor, Green & Cori (1948) was used to prepare aldolase from rabbit skeletal muscle. Enzymic activity was determined in a Beckman DK2 recording spectrophotometer at 30°C by the method of Blostein & Rutter (1963), a specific activity of 16 units/mg commonly being found for the purified enzyme. Protein was estimated spectrophotometrically, taking $E^{1\%}_{1cm}$ as 9.1 at 280nm (Baranowski & Niederland, 1949), or by the method of Lowry, Rosebrough, Farr & Randall (1951) standardized against the spectrophotometric procedure. A mol.wt. of 160000 for the native enzyme and a subunit mol.wt. of 40000 (Kawahara & Tanford, 1966) were assumed throughout.

Chemical modification of native aldolase. Alkylation of thiol groups in the native enzyme was carried out at room temperature (20°C) in 20mM-*N*-ethylmorpholine–acetic acid buffer, pH8.0. Reaction mixtures contained 10mg of protein/ml, 20mM-iodoacetamide or sodium iodoacetate or 2mM-NEM. The concentration of FDP, when present, was 20mM. Reactions were stopped after the required time by adding 2-mercaptoethanol to a final concentration of 100mM, and the alkylated protein was then purified by dialysis against 20mM-*N*-ethylmorpholine–acetic acid buffer, pH8.0, for 24h at 2°C. After dialysis, samples were taken for measurement of enzymic activity and assay with DTNB.

Complete alkylation of enzyme thiol groups. Solid urea to a final concentration of 8M was added to solutions (20mg/ml) of native aldolase or partially alkylated aldolase in 0.1M-tris–HCl buffer, pH8.0. The final protein concentration was about 10mg/ml. A two-fold molar excess of 2-mercaptoethanol over the maximum number of thiol groups present was then added and the tube was flushed with N_2. After 15min a five-fold molar excess of iodo[2-^{14}C]acetic acid neutralized with NaOH was added. The solutions were again flushed with N_2 and then left in the dark at room temperature (20°C) for 50min, after which time they were dialysed against glass-distilled water at 2°C for 24h and freeze-dried. Paper electrophoresis at various pH values of an acid hydrolysate of carboxymethylated aldolase showed that all the radioactivity was accounted for by *S*-carboxymethylcysteine after acid hydrolysis.

Reaction with DTNB. The reaction of the enzyme with DTNB at 30°C in 20mM-*N*-ethylmorpholine–acetic acid buffer, pH8.0, was followed in a Beckman DK2 recording spectrophotometer by measuring the increase in E_{412} resulting from the release of the 5-thio-2-nitrobenzoate anion, for which an extinction coefficient of 1.36×10^4 M^{-1} cm^{-1} was assumed (Ellman, 1959). Reaction was initiated by the addition of DTNB to a final concentration of 2mM. Enzyme concentrations in the range 1–4μM were used, and pseudo-first-order kinetics were observed. The number of groups of a given reactivity and pseudo-first-order rate constants were calculated as described by Freedman & Radda (1968). To test the effect of substrate, the reactions were also carried out in the presence of 4mM-FDP.

Radioactivity measurements. Samples of the tryptic digests of proteins alkylated with iodo[2-^{14}C]acetic acid were plated on to planchets, dried *in vacuo* and radioactivity was counted in a Nuclear–Chicago end-window gas flow counter. For monitoring column effluents, samples (10μl) of each fraction were added to 3ml of toluene–Triton (2:1, v/v) containing 2,5-diphenyloxazole (0.5%, w/v) and counted in a Nuclear–Chicago Unilux scintillation counter.

Amino acid analysis. Samples of proteins and peptides were hydrolysed for 24h at 105°C with 6M-HCl in sealed evacuated tubes. The HCl was removed *in vacuo* over fresh NaOH pellets and the amino acids were analysed on a Beckman 120C automatic amino acid analyser. The values for serine and threonine were corrected, by 6% and 3% respectively, for destruction during 24h of hydrolysis (Perham, 1967).

Digestion with trypsin. Proteins (10mg/ml) in 0.5% NH_4HCO_3, pH8.0, were digested with trypsin (0.1mg/ml) for 6h at 37°C. The buffer was then removed by freeze-drying.

Separation of peptides. Preparation of peptide 'maps' and radioautographs was carried out as described by Harris & Perham (1965). Details of the buffers used for high-voltage paper electrophoresis were as given by Perham (1967). Paper chromatography was done in the descending system (butan-1-ol–acetic acid–water–pyridine, 15:3:12:10, by vol.) of Waley & Watson (1953). Peptides were detected on paper by means of the ninhydrin–cadmium reagent of Heilmann, Barrollier & Watzke (1957) or the chlorination method of Rydon & Smith (1952). Tryptophan-containing peptides were revealed on paper by their fluorescence under u.v. light and the *p*-dimethylaminobenzaldehyde stain described by Dalgleish (1952).

For preparative separation of radioactive tryptic peptides, 100mg of a tryptic digest of *S*-carboxymethylated aldolase were applied to a column (140cm × 2.5cm) of Sephadex G-25 in 0.05M-NH_3. The column was developed at 50ml/h and fractions (6ml) were collected. Samples (10μl) of each fraction were counted in a Nuclear–Chicago Unilux scintillation counter. The radioactive peptides were then freeze-dried and purified further by

paper chromatography followed by high voltage paper electrophoresis at pH6.5 and pH3.5. Peptides were eluted from the paper with 0.05M-NH_3.

Cyanogen bromide cleavage of aldolase. Cyanogen bromide cleavage of *S*-carboxymethylated aldolase and separation of the resulting fragments by gel filtration on Sephadex G-75 were carried out as described by Lai (1968). The column dimensions were 120cm × 2cm and the buffer used was 0.1M-pyridine in 30% (v/v) acetic acid.

N-terminal analysis of peptides. The *N*-terminal residues of peptides were determined by the dansyl method of Gray & Hartley (1963). DNS-amino acids were identified chromatographically on polyamide thin-layer sheets (Woods & Wang, 1967).

RESULTS

A radioautograph of the tryptic peptide 'map' of rabbit muscle aldolase which had been carboxymethylated with iodo[2-^{14}C]acetic acid in the presence of 8M-urea is shown in Fig. 1. Six major radioactive spots were observed (TR2–TR7), together with two additional spots of somewhat lower intensity in the acidic region of the 'map' (TR1*a*, TR1*b*). A small amount of 'core' material also remained after tryptic digestion, giving rise to a radioactive streak from the origin of the 'map' after paper chromatography.

To characterize them further, the radioactive carboxymethylcysteine-containing tryptic peptides were isolated and analysed. The tryptic digest was first fractionated by gel filtration on Sephadex G-25 in 0.05M-ammonia (Fig. 2). The radioactive peaks were freeze-dried and purified further by successive paper chromatography and paper electrophoresis at pH6.5 and pH3.5. Samples of the purified peptides were hydrolysed and Table 1 shows the amino acid compositions obtained. The material designated 'core' in the gel filtration step gave simply an elongated streak in paper chromatography as in the peptide 'maps', did not migrate in paper electrophoresis, and further attempts to purify it have not yet been successful. It is apparent that the compositions of peptides TR1*a* and TR1*b* differ only in the arginine residue present in peptide TR1*b* and absent from peptide TR1*a*. The otherwise identical compositions suggest that these peptides arise from a region of the primary structure in which there is an arginine residue adjacent to and on the *C*-terminal side of a lysine residue. Tryptic hydrolysis would then be expected to lead to the production of two peptides differing only in the presence of an additional arginine residue at the *C*-terminal end of one peptide.

Thus the compositions of the carboxymethylcysteine-containing peptides indicate that they represent seven unique sequences around thiol groups. The somewhat low recoveries of tyrosine and carboxymethylcysteine are not uncommon in peptides eluted from paper. However, the similar specific radioactivities of the radioactive carboxymethylcysteine-containing peptides eluted from the tryptic peptide 'map' are consistent with the presence of only one carboxymethylcysteine residue per peptide. On the other hand, the possibility of an additional tyrosine residue occurring in peptides TR1*a*, TR1*b*, TR2 and TR3, although unlikely, cannot be entirely excluded until the amino acid sequences of the peptides have been established.

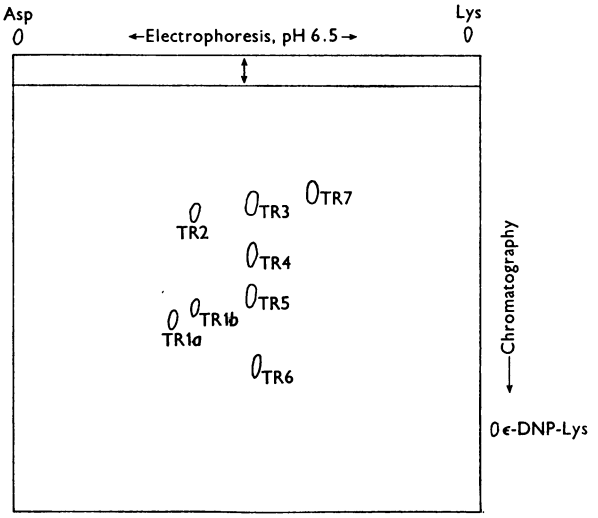

Fig. 1. Radioautograph of a tryptic peptide 'map' of aldolase carboxymethylated in 8M-urea with iodo[2-^{14}C]-acetic acid, separated by electrophoresis at pH6.5 followed by chromatography. For other details, see text.

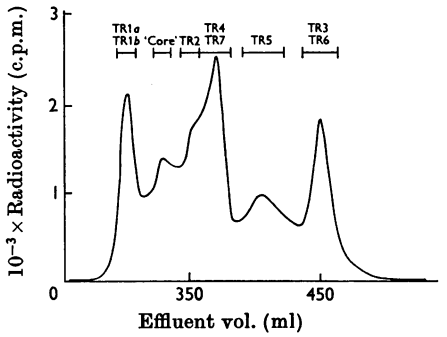

Fig. 2. Separation of the tryptic peptides of *S*-carboxymethylated aldolase by gel filtration on Sephadex G-25. The column was 140cm × 2.5cm and the buffer used was 0.05M-NH_3. Samples (10μl) of each fraction were counted. The radioactive peaks were pooled and freeze-dried as shown. The peptides subsequently found in each peak are indicated. For other details, see the text.

Table 1. *Amino acid compositions of the carboxymethylcysteine-containing tryptic peptides of S-carboxymethylated aldolase*

The peptides were purified as described in the text. Compositions are given as mol/mol of peptide. CMCys, carboxymethylcysteine.

Peptide ...	TR1*a*	TR1*b*	TR2	TR3	TR4	TR5	TR6	TR7
Amino acid								
Lys	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.93
His	0.97	1.03						0.97
Arg		1.05						
CMCys	0.43	0.61	0.75*	0.72*	0.53	0.62*	0.69*	0.84
Asp	3.83	3.98			1.00			
Thr			0.89					1.90
Ser	1.06	0.95			0.86			
Glu	3.97	4.11	1.96	1.01	1.05			2.00
Pro	2.76	2.86					1.96	1.00
Gly	2.03	2.13			1.02			1.05
Ala	0.98	0.98		0.99	2.71*			1.00
Val	1.22	1.29	0.91			0.91		1.00
Met								0.65*
Ile	2.79	2.95						
Leu	1.87	2.02			1.92	0.99	2.03	
Tyr	0.63*	0.83*	0.58	0.50				
Phe								
Trp							+	

* *N*-Terminal residue established by the dansyl technique.

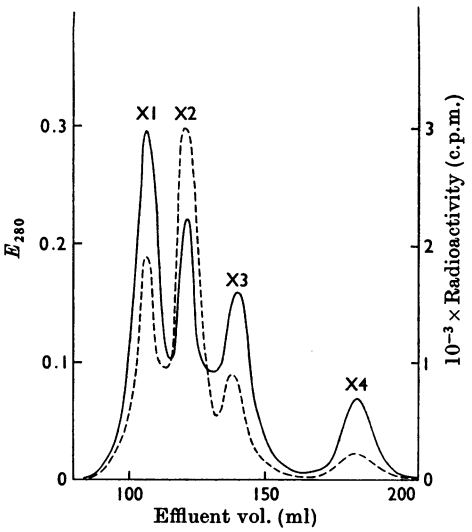

Fig. 3. Elution profile of the cyanogen bromide peptides of [^{14}C]carboxymethylated aldolase on Sephadex G-75. ——, c.p.m. in samples (10μl); ----, E_{280}. For other details, see the text.

Fig. 3 shows the separation by gel filtration on Sephadex G-75 of the peptides produced by cyanogen bromide cleavage of *S*-carboxymethylated aldolase. Four fragments were isolated, all of which contained radioactivity. Since cyanogen bromide cleavage before tryptic digestion did not alter the positions of the carboxymethylcysteine-containing peptides on the peptide 'maps', it was possible to assign each radioactive tryptic peptide to a cyanogen bromide cleavage fragment by examining tryptic peptide 'maps' of the separated fragments. These assignments are given in Table 2.

The effect of various thiol-blocking reagents on the activity of aldolase is given in Table 3. It can be seen that the ability to inactivate under the conditions described is in the order NEM> iodoacetamide>iodoacetic acid. In all cases, the presence of the substrate, FDP, decreased the rate of inactivation, but prolonged exposure to the alkylating reagent inactivated the enzyme even in the presence of substrate.

After alkylation of the native enzyme with various reagents for various lengths of time, the reaction of the thiol groups of the chemically modified enzyme with DTNB was studied. The results are given in Table 4, expressed per subunit of mol.wt. 40000. In native aldolase one thiol group per subunit reacted with DTNB very quickly, with a rate constant too large to be measured by the method used. A further set of two other thiol groups reacted more slowly, with rates that were either identical or very similar. These results are in good agreement with those of Eagles *et al.* (1969).

Table 2. *Location of S-carboxymethylcysteine-containing peptides within the cyanogen bromide fragments of aldolase*

The designations of carboxymethylcysteine-containing peptides and cyanogen bromide fragments are given in Figs. 1 and 2 respectively.

Fragment ...	X1	X2	X3	X4
Peptide ...	TR3, TR5, 'core'	TR4, TR6	TR1*a*, TR1*b*, TR2	TR7

Table 3. *Effect of time of exposure to thiol-blocking reagents on the activity of aldolase*

Results are expressed as percentage of controls incubated for an identical time in the presence or absence of FDP. The presence of FDP decreased the control specific activity by about 10% in 44h. For other details, see the text.

	Enzymic activity remaining			
Time of reaction (h) ...	1	4	24	44
Reagent				
NEM	50	13	0	—
NEM+FDP	100	60	34	—
Iodoacetamide	100	87	21	—
Iodoacetamide +FDP	100	96	78	—
Iodoacetic acid	100	100	50	40
Iodoacetic acid +FDP	100	100	100	69

Treatment of the enzyme (10mg/ml) with 20mM-FDP for 24h followed by removal of the substrate by dialysis before the DTNB reaction was carried out caused some modification of the enzyme that resulted in decreased reactivity of the thiol groups of the slow set (Table 4). This effect is not the same as that observed with untreated enzyme when the reaction with DTNB was carried out in the presence of 4mM-FDP. In that case one of the thiol groups of the slow set was protected, whereas the other reacted at the same rate as in the native enzyme and the reaction of the single fast group was unaffected.

After short exposure (2h) to iodoacetamide, only one thiol group per subunit reacted with DTNB, and the reactivity of this group was approximately twice that of the thiol groups of the slow set in the native enzyme (Table 4). This remaining group did not react with DTNB when the DTNB reaction was carried out in the presence of 4mM-FDP. Prolonged exposure (24h) to iodoacetamide in the absence of FDP abolished all reaction of aldolase with DTNB. However, the presence of 20mM-FDP during the alkylation with iodoacetamide served to protect one thiol group which could subsequently react with DTNB, although the incubation with substrate again appeared to have altered the rate constant (Table 4). Similar results were obtained for treatment of aldolase with NEM, but shorter times of reaction served to give the same effects as iodoacetamide (cf. Table 3). In addition, even after prolonged treatment (44h) of the native enzyme with iodoacetic acid, one slowly reacting thiol group remained reactive towards DTNB.

Radioautographs were prepared of tryptic peptide 'maps' of aldolase that had been treated with iodoacetamide for 24h in the absence and presence of 20mM-FDP, followed by complete alkylation of the unchanged thiol groups with iodo[2-^{14}C]acetic acid in 8M-urea. It was found that after treatment (24h) with iodoacetamide, radioactive peptides TR4, TR6 and TR7 (Fig. 1) were missing. In the presence of FDP, however, peptides TR6 and TR7 were lost but peptide TR4 remained. Treatment (2h) of the native enzyme with iodoacetamide, and subsequent complete alkylation with iodo[2-^{14}C]acetic acid in 8M-urea, also resulted in the loss of only radioactive peptides TR6 and TR7 from the radioautograph whether FDP was present or not during the alkylation of the native enzyme. The reaction of NEM with aldolase was very similar to that of iodoacetamide. Thus treatment of the native enzyme with NEM for 1h before complete alkylation with iodo[2-^{14}C]acetic acid in 8M-urea caused the loss of radioactive peptides TR4, TR6 and TR7, whereas the presence of 20mM-FDP during the 1h reaction with NEM led to the loss of peptides TR6 and TR7 only. On the other hand, even prolonged incubation (44h) of native aldolase with iodoacetic acid before complete alkylation with iodo[2-^{14}C]acetic acid in 8M-urea did not cause peptide TR4 to disappear entirely from the radioautographs, and pretreatment (24h) with non-radioactive iodoacetic acid was required before peptides TR6 and TR7 disappeared completely. Thus the results of peptide 'mapping' of variously alkylated aldolases are in good agreement with the results of DTNB reaction (Table 4) in showing that only three thiol groups are available for reaction in the native enzyme and that one may be protected by the presence of substrate.

Table 4. *Effect of modification of aldolase with iodoacetamide on thiol group reactivity towards DTNB*

Reactions with DTNB were carried out at 30°C in 20mM-*N*-ethylmorpholine–acetic acid buffer, pH 8.0. Enzyme concentrations of 1–4 μM were used; the reaction was initiated by adding DTNB to a final concentration of 2mM. For other details, see the text.

Nature of aldolase pretreatment	Time of treatment (h)	No. of groups reacting/40000-mol.wt. subunit		k (min^{-1})	Half-life (min)
		Fast set	Slow set	Slow set	Slow set
None	—	0.71	1.96	0.399±0.02	1.7
FDP	2	0.77	1.61	0.083±0.02	8.1
FDP	24	0.87	2.02	0.091±0.02	7.4
Iodoacetamide	2	0	0.93	0.825±0.02	0.81
Iodoacetamide	24	0	0	—	—
Iodoacetamide+FDP	2	0	1.10	0.123±0.02	5.4
Iodoacetamide+FDP	24	0	0.96	0.091±0.02	7.4

DISCUSSION

Recent results indicate that the molecular weight of rabbit muscle aldolase is 160000 and that the enzyme dissociates into four subunits of mol.wt. 40000 in the presence of guanidine hydrochloride (Kawahara & Tanford, 1966). Independent evidence that aldolase is tetrameric has been obtained from hybridization studies (Penhoet, Kochman, Valentine & Rutter, 1967). It is generally assumed that the tetramer is composed of two subunits of one type of polypeptide chain and two subunits of another type of polypeptide chain (Morse & Horecker, 1968), although part of the evidence on which the claim of two types of chain was based suggested that they were present in unequal amounts (Chan *et al.* 1967).

Several studies (Morse & Horecker, 1968; Eagles *et al.* 1969; Anderson *et al.* 1969) have indicated a total of approximately 28 thiol groups per tetramer of mol.wt. 160000. If the reported compositions (Chan *et al.* 1967) of the two types of protein subunit are correct, these thiol groups are unequally distributed between the two types of chain. However, a comparative study of the structure of aldolases isolated from a variety of sources has suggested that the two types of protein subunit must be very similar, if not identical, in primary structure (Anderson *et al.* 1969). In particular, there was evidence of an equal distribution of cysteine residues between the two types of polypeptide chain. This is also more in accord with the study of the cyanogen bromide cleavage of rabbit muscle aldolase (Lai, 1968).

No evidence of unequal distribution of cysteine residues between the two types of protein chain was obtained in the present study. In the absence of denaturing reagents, three specific cysteine residues could be modified with NEM and iodoacetamide and the presence of substrate abolished the reaction of a specific thiol group, that of peptide TR4, with these reagents. The reaction of DTNB with aldolase modified by alkylation with NEM or iodoacetamide indicates that DTNB reacts with the same cysteine residues as NEM and iodoacetamide, and the numbers of thiol groups involved in the reactions suggest that these three groups are common to all four subunits. Since, within the limits of the method used, pseudo-first-order kinetics are observed for the DTNB reaction, the simplest interpretation is that corresponding thiol groups in different subunits are identical and that no subunit interaction has been detected. Seven unique carboxymethylcysteine residues have been found in tryptic digests of aldolase carboxymethylated with iodo[2-^{14}C]acetic acid in 8M-urea. Thus the total number of carboxymethylcysteine-containing tryptic peptides, the results of peptide 'mapping' of variously alkylated aldolases and the amino acid composition of *S*-carboxymethylated aldolase (Anderson *et al.* 1969) are consistent with the presence of at least four thiol groups in each subunit that do not react in the absence of protein denaturing agents. Further amino acid sequence work on the isolated peptides is required to establish this point unequivocally since the significance of small amounts of a radioactive 'core' in the present experiments is not clear and the presence of an additional thiol group in the aldolase subunit cannot therefore be rigidly excluded. For the moment, however, it seems reasonable to assume that there are 28 (or possibly 32) cysteine residues in aldolase distributed equally between four subunits, and that the position and reactivity of a given residue is identical in all four subunits.

It is apparent that loss of activity accompanying the modification of thiol groups is associated with the modification of the cysteine residue located in peptide TR4. Thus it was possible to modify the cysteine residues in peptides TR6 and TR7 without

modifying the residue in peptide TR4. This could be achieved either by using only brief exposure of the native enzyme to alkylating reagent or by protecting this thiol group with substrate during longer exposures. No loss of activity was observed until the thiol group of peptide TR4 was modified. However, since it was impossible by the methods used to modify this residue without simultaneously modifying the thiol groups in peptides TR6 and TR7, it is not clear whether modification of the thiol group of peptide TR4 is in itself sufficient to inhibit the enzyme.

It is important to try and correlate the thiol groups reacting with DTNB (Table 4) with those reacting with the various alkylating reagents examined. It seems clear that the cysteine residue of peptide TR4 is one of two groups in the slow set for the DTNB reaction, since its reactivity with DTNB in the enzyme partially alkylated (2h) with iodoacetamide is comparable with that of the slow set in the native enzyme and very different to that of the fast group (Table 4). The observation that its reactivity in the alkylated enzyme is, in fact, approx. twice that in the native enzyme suggests a protein conformational change accompanying reaction of the thiol groups of peptides TR6 and TR7 with iodoacetamide. It is unlikely that this conformational change can be large, since the enzymic activity is scarcely affected (Table 3). To distinguish the thiol groups of peptides TR6 and TR7 is, perhaps, more difficult. That of peptide TR7 may be recognized from its surrounding amino acids as the thiol group adjacent in the primary structure to the lysine residue that forms a Schiff base with the substrate (Lai, Hoffee & Horecker, 1965). It is tempting, therefore, to suppose that it resides near the surface of the enzyme and, in consequence, is the thiol group that reacts rapidly with DTNB. The other member of the slow set would then have to be the cysteine residue of peptide TR6. These experiments also show clearly that the thiol groups of aldolase can be distinguished by their rates of reaction with the various reagents. Thus two thiol groups react rapidly with iodoacetamide and NEM (peptides TR6 and TR7) and one reacts slowly (peptide TR4) whereas one reacts rapidly with DTNB (probably peptide TR7) and two react slowly (peptides TR4 and TR6).

The partial modification of aldolase with iodoacetamide might provide a method of introducing a heavy metal into the active site of aldolase for X-ray crystallographic analysis. After exposure (2h) to iodoacetamide, two thiol groups (peptides TR6 and TR7) are alkylated, whereas that of peptide TR4 is unmodified and enzymic activity is retained. Since this thiol group exhibits substrate protection towards modification, it is possible that it is at or near the active site. Reaction of the enzyme with iodoacetamide for 2h under the conditions described, followed by treatment with a suitable heavy-atom-containing reagent could therefore result in specific introduction of the heavy atom at a cysteine residue in the active site of aldolase.

Amino acid sequence studies have shown that the order (from the *N*-terminus) of the cyanogen bromide cleavage fragments of aldolase is X1-X3-X4-X2 and that the lysine residue that forms a Schiff base with substrate and is considered to be in the active site is located in fragment X3 (Lai, 1968). The present study shows that the substrate-protected thiol group (peptide TR4) of aldolase is in fragment X2 (Table 1). It is clear therefore that the two residues are far apart in the primary structure and, assuming that the cysteine residue of peptide TR4 is in the active site of aldolase, it is apparent that the active site must be formed by extensive folding of the polypeptide chain.

Inhibition of the enzyme resulting from prolonged incubation with excess of substrate has been observed by others (Woodfin, 1967; Lai, Martinez-de Dretz, Bacila, Marinello & Horecker, 1968). We find that this inhibition is accompanied by decreased thiol group reactivity towards DTNB (Table 3). If the decrease in reactivity were simply due to substrate still bound to the enzyme after dialysis, it would be expected that under conditions that would remove all FDP from the reaction mixture, the reactivity of the thiol groups towards DTNB would revert to that observed in the absence of substrate. However, addition of the coupling enzymes, glyceraldehyde 3-phosphate dehydrogenase and triose phosphate isomerase, and NADH, had no effect on the reactivity of the thiol groups of aldolase which had been exposed to FDP. It therefore appears that substrate is capable of reacting with aldolase to produce some modified form of the enzyme. Experiments with radioactive FDP are probably required to demonstrate the nature of this reaction.

Finally, it has already been mentioned that peptide TR7 may be recognized as occurring in the published amino acid sequence around the lysine residue that forms a Schiff base with the substrate (Lai *et al.* 1965). If that sequence is correct, peptide TR7 must be generated by cleavage of a Pro-Met bond during the tryptic hydrolysis of the protein since peptide TR7 has methionine as its *N*-terminal residue (Table 1). Such a cleavage by trypsin or even a chymotrypsin-like contaminant of trypsin is most unusual and suggests that the amino acid sequence in this region of the molecule deserves reinvestigation.

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